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(54) Title: VIRAL VECTORS WITH LATE TRANSGENE EXPRESSION

(57) Abstract

The present invention is directed to recombinant viruses containing a therapeutic transgene operably linked to a late regulatory element. The vectors of the present invention are capable of replication and lysis of neoplastic cells. The vectors may optionally include modifications to the genome so as to impart additional therapeutic, conditionally replicating or targeting functions. The present invention also provides pharmaceutical formulations of such vectors. The present invention further provides methods of use of such vectors. The present invention further provides methods of preparing the vectors.

TITLE

VIRAL VECTORS WITH LATE TRANSGENE EXPRESSION

BACKGROUND OF THE INVENTION

The genome of the adenovirus has been well characterized. This information has been used to design recombinant adenoviruses capable of acting as vectors for the introduction of exogenous DNA into target cells. Many of these viral vectors contain modifications to the early gene products to endow the vectors with specific activities. For example, the early genes E1 and E2, are transcribed early after infection. The products of these genes are responsible for the suppressing the ability of the infected cell to respond to the infection and to promote viral replication in the host.

In particular, the immediate early gene E1a is transcribed rapidly following infection. The E1a product can immortalize primary cells *in vivo*. E1a has also been show to bind a cellular protein p105 RB, the product of the retinoblastoma gene. The E1b gene is not capable of transforming cells on its own, but cooperates with E1a to stably transform cells. Both E1a and E1b are necessary for the full transformation and tumor formation in animals. The E1a gene has a variety of functions. The full scope of activity of the adenovirus E1a region is described in Bayley, S. and Mymryk, J. (1994) Intl. J. of Oncology 5:425-444.

The E1b genes are known to interact with host cell proteins. In particular two proteins are produced by differential splicing of the E1b sequence, p19 and p55. The p55 protein has been well characterized as interacting with the p53 gene product. p53 is a well characterized protein and has been shown to activate programmed cell death (PCD) apoptotic pathways when present in sufficient intracellular concentration. p53 induced apoptosis has been shown to take place in response to a wide variety of cell injury including radiation, DNA damaging agents, etc. By binding to p53, p55 prevents the formation of the active p53 phosphorylated tetramer. Since p53 is sequestered by p55, a primary apoptotic pathway is never initiated and the infected cell undergoes uncontrolled replication in combination with Rb activity in response to the E1a 12S and 13S proteins.

required. This is favorable from an immunological perspective as well as for economic reasons in the manufacture of such agents.

In order to facilitate the understanding of the present invention, a brief overview of the life cycle of a typical virus used for delivery of exogenous transgenes, the adenovirus, is offered. The adenoviral replicative cycle in human cells can be divided into the early and late phase which are punctuated by the onset of viral DNA replication. The early phase beings when viral particles attach to cells through interaction between the virion fiber domain and cell surface receptors. The virion moves into the cell by either endocytosis or direct penetration of the cytoplasmic membrane and is transported to the nucleus where most of the capsid is shed. In the nucleus, the virion core proteins are removed yielding viral chromosomes that are almost entirely devoid of virion proteins. Expression of the viral genome is temporally coordinated and begins with the E1a region about one hour after infection. The other early genes E1b, E2, E3 and E4 are first express soon after E1a at the 1.5-2.0 hours post infection, A number of the protein products encoded by the early genes are required for viral DNA replication, while other prepare the DNA synthesis machinery of the infected cell for efficient viral DNA replication. Some early virally encoded proteins have been associated with protecting infected cells from immune surveillance.

The late phase of infection with onset of DNA replication at about 7 hour post infection. In the native adenovirus, the messenger RNAs for all late gene products are spliced from a primary RNA which is transcribed from the major late promoter (MLP). The MLP is located at position 16.5 on the r-strand. Although the major late promoter is active to a limited extent in the early phase of infection, the transcription does not proceed past map position 39. During the late phase of the viral life cycle, the MLP is fully activated and continues to map position 99. Each late primary RNA transcript is processed into one of five different mRNAs, L1-L5. These mRNAís all contain a common tripartite leader sequence of 203 nucleotides. Late mRNAs encode capsid components and proteins required for assembly of virions and packaging of the viral chromosome. Viral DNA replication requires the terminal protein for initiation and proceed by a semi-conservative mechanism. With the onset of replication, efficient transcription of the late gene families from the major late promoter begins and attains a maximal level approximately 18 hours post infection. During the late phase viral

cancer cells according to the procedures of Example 2.c. herein. Again, the time course experiment demonstrates the temporal expression of p53 in the replication competent MLP-p53 construct.

Figure 4 presents the results of an experiment similar to that presented in Figure 3 except that a replication competent E1bdl55k-CMV-p53 virus was included for comparison and only a single dose was administered according to the procedures of Example 2.d. herein. This time course experiment demonstrates the temporal expression of p53 in the replication competent MLP-p53 construct as compared to the substantially similar construct wherein the p53 gene was under control of the constituitive CMV promoter. This data confirms that the MLP-p53 construct is indeed expressing the p53 in a temporal manner late in the viral replication cycle.

Figure 5 is a digest of viral DNA from SK-BR3 cells infected with a one hour pulse of the indicated viruses at a concentration of 1.8×10^9 particles/ml and harvested approximately 48 hours later according to the procedures of Example 2.d. herein. The results presented demonstrate that replication competent wild-type Ad5 (Ad5WT), replication competent E1Bdl55K (ZAZA) virus and replication competent E1Bdl55K-MLP-p53 (55K/MLP53) virus all replicate their viral DNA well while the replication deficient adenovirus control (rAdcon) and the replication deficient vector encoding p53 (FTCB) does not.

Figure 6 is a graphical representation of the data obtained *in vivo* in a PC-3 mouse tumor model. Tumor volume is plotted on the vertical axis and days following administration is plotted on the horizontal axis. As can be seen from the data presented, the replicating virus E1Bdl55K-MLP-p53 (cFAMA) was able to produce tumor regression in an *in vivo* mouse model of human cancer. The replication competent CMV driven p53 virus also replicates its viral DNA, but to a lesser extent.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a replication competent recombinant virus containing a therapeutic transgene operably linked to a late regulatory element.

I. Replication Competent Recombinant Virus:

The term "replication competent" is made in reference to a virus which is capable of replicating its genome and packaging the replicated viral genome into

thereof the individual serotypes and A-F subgenera including but not limited to human adenovirus types 1, 2, 3, 4, 4a, 5, 6, 7, 8, 9, 10, 11 (Ad11A and Ad 11P), 12, 13,14,15,16,17,18,19, 19a, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 34a, 35, 35p, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, and 91. The term bovine adenoviruses includes but is not limited to bovine adenovirus types 1,2,3,4,7, and 10. The term canine adenoviruses includes but is not limited to canine types 1 (strains CLL, Glaxo, RI261, Utrect, Toronto 26-61) and 2. The term equine adenoviruses includes but is not limited to equine types 1 and 2. The term porcine adenoviruses includes but is not limited to porcine types 3 and 4. The term recombinant adenovirus also includes chimeric (or even multimeric) vectors, i.e. vectors constructed using complementary coding sequences from more than one viral subtype. See, e.g. Feng, *et al.* Nature Biotechnology 15:866-870.

In the preferred practice of the invention, the recombinant adenoviral vector is derived from genus adenoviridiae. Particularly preferred viruses are derived from the human adenovirus type 2 or type 5. In the preferred practice of the invention as exemplified herein, the preferred vector is derived from the human adenoviridiae. More preferred are vectors derived from human adenovirus subgroup C. Most preferred are adenoviral vectors derived from the human adenovirus serotypes 2 and 5. In the most preferred practice of the invention the virus is derived human adenovirus Type 5 dl309. dl327, dl520 or wild-type adenovirus.

II. Late Regulatory Element

The term "late regulatory element" refers to regulatory element which drives transcription of the therapeutic transgene a point later in time than the element which induces initial viral replication. Characteristically, these promoter elements are found driving expression of packaging proteins and other proteins late in the viral life cycle. An example of such late regulatory element when the parent vector is adenovirus is the adenovirus major late promoter (MLP). Other viral vector systems also possess late temporally regulated promoters. For baculoviral vectors, the AcNPV basic gene promoter and the polyhedrin gene promoters may be employed (Sridhar, *et al.* (1993) FEBS Lett. 315:282-286. For herpes simplex viruses, the Latent Activated Promoters may be employed. See, e.g. Rivera-Gonzalez, *et al.* (1994) Virology 202:550-564 and Imbal, *et al.* (1992) J. Virol. 66:5453-5463. For human papilloma viruses, the

(1987) Nature 329:642), the MMAC-1 gene, the adenomatous polyposis coli protein (Albertsen, *et al*., United States Patent 5,783,666 issued July 21, 1998), the deleted in colon carcinoma (DCC) gene, the MMSC-2 gene, the NF-1 gene, nasopharyngeal carcinoma tumor suppressor gene that maps at chromosome 3p21.3. (Cheng, *et al*. 1998. Proc. Nat. Acad. Sci. 95:3042-3047), the MTS1 gene, the CDK4 gene, the NF-1 gene, the NF2 gene, and the VHL gene.

B. Antigenic Genes

The term "antigenic genes" refers to a nucleotide sequence, the expression of which in the target cells results in the production of a cell surface antigenic protein capable of recognition by the immune system. Examples of antigenic genes include carcinoembryonic antigen (CEA), p53 (as described in Levine, A. PCT International Publication No. WO94/02167 published February 3, 1994). In order to facilitate immune recognition, the antigenic gene may be fused to the MHC class I antigen.

C. Cytotoxic Genes

The term "cytotoxic gene" refers to nucleotide sequence, the expression of which in a cell produces a toxic effect. Examples of such cytotoxic genes include nucleotide sequences encoding pseudomonas exotoxin, ricin toxin, diptheria toxin, and the like.

D. Cytostatic Genes

The term "cytostatic gene" refers to nucleotide sequence, the expression of which in a cell produces an arrest in the cell cycle. Examples of such cytostatic genes include p21, the retinoblastoma gene, the E2F-Rb gene, genes encoding cyclin dependent kinase inhibitors such as P16, p15, p18 and p19, the growth arrest specific homeobox (GAX) gene as described in Branellec, *et al*. (PCT Publication WO97/16459 published May 9, 1997 and PCT Publication WO96/30385 published October 3, 1996).

E. Cytokine Gene

The term "cytokine gene" refers to a nucleotide sequence, the expression of which in a cell produces a cytokine. Examples of such cytokines include GM-CSF, the interleukins, especially IL-1, IL-2, IL-4, IL-12, IL-10, IL-19, IL-20, interferons of the

NGF, VEGF (to increase blood perfusion to target tissue, induce angiogenesis, PCT publication WO98/32859 published July 30, 1998), thrombospondin etc.

H. Pro-Apoptotic Genes:

The term "pro-apoptotic gene" refers to a nucleotide sequence, the expression thereof results in the programmed cell death of the cell. Examples of pro-apoptotic genes include p53, adenovirus E3-11.6K, the adenovirus E4orf4 gene, p53 pathway genes, and genes encoding the caspases. The p16 gene is also apoptotic in Rb positive, p16 negative, p53 wild type tumors. (Frizelle, *et al*. (1998) Oncogene 16:3087-95 and Sandig, *et al.* (1997) Nature Medicine 3:313)

I. Pro-Drug Activating Genes:

The term "pro-drug activating genes" refers to nucleotide sequences, the expression of which, results in the production of protein capable of converting a non-therapeutic compound into a therapeutic compound, which renders the cell susceptible to killing by external factors or causes a toxic condition in the cell. An example of a prodrug activating gene is the cytosine deaminase gene. Cytosine deaminase converts 5-fluorocytosine (5-FC) to 5-fluorouracil (5-FU), a potent antitumor agent. The lysis of the tumor cell provides a localized burst of cytosine deaminase capable of converting 5FC to 5FU at the localized point of the tumor resulting in the killing of many surrounding tumor cells. This results in the killing of a large number of tumor cells without the necessity of infecting these cells with an adenovirus (the so-called bystander effect"). Additionally, the thymidine kinase (TK) gene (see e.g. Woo, *et al.* United States Patent No. 5,631,236 issued May 20, 1997 and Freeman, *et al.* United States Patent No. 5,601,818 issued February 11, 1997) in which the cells expressing the TK gene product are susceptible to selective killing by the administration of gancyclovir may be employed.

J. Anti-Angiogenic Genes:

The term "anti-angiogenic" genes refers to a nucleotide sequence, the expression of which results in the extracellular secretion of anti-angiogenic factors. Anti-angiogenesis factors include angiostatin, inhibitors of vascular endothelial growth factor (VEGF) such as Tie 2 (as described in PNAS(USA)(1998) 95:8795-8800), and endostatin.

Research News Briefs. Nature Biotechnology. 15:205:1997; O'Hare, *et al.* PCT publication WO97/05265 published February 13, 1997. A similar targeting moiety derived from the HIV Tat protein is also described in Vives, *et al.* (1997) J. Biol. Chem. 272:16010-16017.

V. Additional Modifications to the Virus:

The present invention also provides recombinant adenoviruses containing additional modifications to the viral genome such as targeting modifications, modifications to make the vectors replicate selectively in particular cell types or phenotypic states, controlled expression characteristics, suicide genes or additional modifications to enhance cytotoxicity. However, this is not meant to imply that other modifications to the viral genome may not also be included or that multiple such modifications may not be employed.

A. Selectively Replicating:

The term "selectively replicating" refers to a recombinant viral vector capable of preferential replication in one cell type versus another cell type, in a cell in one phenotypic state relative to another phenotypic state, or in a given cell type in response to an external stimuli. Selective replication is achieved by the use of replication control elements. The term "replication control elements" refers to DNA sequences inserted into the viral genome or modifications to the viral genome in order to produce recombinant viral vectors which selectively replicate in one cell type versus another cell type, in a cell in one phenotypic state relative to another phenotypic state (cell state specific), or in a given cell type in response to an external stimuli (inducible). Examples of such replication control elements include cell-type specific promoter, cell state specific promoters, and inducible promoters.

1. Cell Type Specific Promoters:

Cell type specific replication may be achieved by the linkage of a cell type specific promoter to an early viral gene such as the E1, E1a, E2 or E4 gene when the virus is selected from the adenovirus genome. The term "cell type specific promoter" refers to promoters which are differentially activated in as a result of cell cycle progression or in different cell types. Examples of cell-type specific promoters includes cell cycle regulatory gene promoters, tissue specific promoters or pathway responsive

constructed from one or more copies of a sequence that matches a consensus binding motif. Such consensus DNA binding motifs can readily be determined. Such consensus sequences are generally arranged as a direct or head-to-tail repeat separated by a few base pairs. Elements that include head-to-head repeats (e.g. AGGTCATGACCT) are called palindromes or inverted repeats and those with tail-to-tail repeats are called everted repeats.

i. Examples Of Pathway Promoters:

Examples of pathway-responsive promoters useful in the practice of the present invention include synthetic insulin pathway-responsive promoters containing the consensus insulin binding sequence (Jacob, *et al.* (1995). J. Biol. Chem. 270:27773-27779), the cytokine pathway-responsive promoter, the glucocorticoid pathway-responsive promoter (Lange, *et al.*(1992) J Biol. Chem. 267:15673-80), IL1 and IL6 pathway-responsive promoters (Won K.-A and Baumann H. (1990) Mol.Cell.Biol. 10: 3965-3978), T3 pathway-responsive promoters, thyroid hormone pathway-responsive promoters containing the consensus motif: 5' AGGTCA 3.', the TPA pathway-responsive promoters (TREs), TGF–β pathway-responsive promoters (as described in Grotendorst, *et al.*(1996) Cell Growth and Differentiation 7: 469-480). Additionally, natural or synthetic E2F pathway responsive promoters may be used. An example of an E2F pathway responsive promoter is described in Parr, *et al.* (1997), Nature Medicine 3:1145-1149 which describes an E2F-1 promoter containing 4 E2F binding sites and is reportedly active in tumor cells with rapid cycling. Examples of other pathway-responsive promoters are well known in the art and can be identified in the Database of Transcription Regulatory Regions on Eukaryotic Genomes accessible through the internet at http://www.eimb.rssi.ru/TRRD.

ii. Preferred Pathway Promoters:

In the preferred practice of the invention as exemplified herein, the vector comprises a synthetic TGF–β pathway-responsive promoter active in the presence of a functional TGF–β pathway such as the promoter containing SRE and PAI pathway responsive promoters. PAI refers to a synthetic TGF–β pathway-responsive promoter comprising sequences responsive to TGF–β signally isolated from the plasminogen activator-I promoter region. The PAI-pathway-responsive promoter may be isolated as a 749 base pair fragment isolatable from the plasmid p800luc (as described in

2. Cell State Specific:

Examples of different phenotypic states would include the neoplastic phenotype versus a normal phenotype in a given cell type. Selective replication is achieved by the use of viral replication control elements. The term viral replication control element refers to a DNA sequence engineered into the vector of the present invention such that the virus is preferentially enabled to replicate the viral genome in a particular type of target cell. Again, these cell state specific promoters may be linked to an early gene such as E1, E2, or preferably E4 to achieve selective replication in response to specific phenotypic states.

a. Tumor Specific:

In order to achieve expression of the adenovirus in tumor cells, one may employ a tumor specific promoter to drive expression of an early gene. The term "tumor specific promoters" refers to promoters which are active in tumor cells and inactive in cells which are not transformed. Examples of tumor specific promoters include the alpha-fetoprotein promoter, the tyrosinase promoter. The use of tumor specific promoters to achieve conditional replication of adenoviral vectors is described in co-pending United States Patent Application 08/433,798 filed May 3, 1995 and International Patent Application No. PCT/US96/06199 published as International Publication No. WO 96/34969 on November 7, 1996 the entire teaching of which is herein incorporated by reference.

For example, the alpha-fetoprotein promoter could be used to replace the endogenous E4 promoter and achieve greater selectivity in conditional replication . Other factors such as NF-IL6 can substitute for E1a in regulating E1a responsive promoters in the adenovirus in the absence of E1a function (Spergel, *et al.* (1992) J. Viroll 66:1021-1030) and this can be avoided by substitution of the E4 promoter with a tumor specific promoter.

b. Repressor of Viral Replication:

Although one may use the pathway responsive promoter to drive replication of the virus in the presence of a functional pathway, alternatively, the one may use a pathway responsive promoter to drive expression of a repressor of viral replication to control expression. The term "repressor of viral replication" refers to a protein, if

3. Inducible Promoters

The term "inducible promoter" refers to promoters which facilitate transcription of the therapeutic transgene preferable (or solely) under certain conditions and/or in response to external chemical or other stimuli. Examples of inducible promoters are known in the scientific literature (See, e.g. Yoshida and Hamada (1997) Biochem. Biophys. Res. Comm. 230:426-430; Iida, *et al*. (1996) J. Virol. 70(9):6054-6059; Hwang, *et al*. (1997) J. Virol 71(9):7128-7131; Lee, *et al*. (1997) Mol. Cell. Biol. 17(9):5097-5105; and Dreher, *et al*. (1997) J. Biol. Chem. 272(46); 29364-29371. Examples of radiation inducible promoters include those induced by ionizing radiation such as the Egr-1 promoter (as described in Manome, *et al.* (1998) Human Gene Therapy 9:1409-17; Takahashi, *et al*. (1997) Human Gene Therapy 8:827-833; Joki. *et al.* Human Gene Therapy (1995) 6:1507-1513; Boothman, *et al.* (1994) volume 138 supplement pages S68-S71; and Ohno, T (1995) Tanpakushitsu Kakusan Koso 40:2624-2630), X-ray inducible promoters such as the XRE promoter (as descrbed in Boothman, *et al*. (1994) Radiation Research 138(Suppl.1):S68-S71), and UV inducible promoters such as those isolated from *Clostridium perfringens* (Garnier and Cole (1988) Mol. Microbiol. 2:607-614.

B. Alternative Modifications to the Viral Genome:

1. E1Bdl55K deletion:

As previously indicated the E1b 55K protein binds to p53. Consequently, in order to enhance the effect of the p53 introduced by the viral vector it is preferred to introduce Descriptions of E1Bdl55K mutations to eliminate p53 binding described in McCormick, United States Patent No. 5,677,178 issued October 14, 1997, the entire teaching of which is herein incorporated by reference.

In the preferred practice of the invention as exemplified herein, the virus is a recombinant adenoviral vector encoding p53 under control of the MLP promoter containing a deletion of nucleotides 2247-3272 of the adenoviral genome to eliminate the function of the E1b 55K protein.

2. E4 Modifications

Addtionally modfications to increase the potency of the vectors of the present invention include but are not limited to alterations within E1b. The vectors of the

approximately 219 to approximately 289 of the E1a 289R protein (or approximately amino acids 173 to approximately amino acid 243 of the E1a 243R protein. In the preferred practice of the invention the deletion of the binding to the p300 family members is achieved by introducing a deletion corresponding to amino acids 4-25 of the E1a 243R and 289R proteins or amino acids 38-60 of the E1a 243R and 289R proteins. In the preferred practice of the invention the deletion of the binding to the pRb family members is achieved amino acids 111-123 of the E1a 243R and 289R proteins. Alternatively, deletion of the binding to the pRb family members may be achieved by eliminate of amino acids 124-127 of the E1a 243R and 289R proteins.

4. E3 Modifications:

The E3 region of the adenovirus encodes proteins which help adenovirally infected cells avoid clearance by the immune system (Wold, *et al.* (1995) Curr. Top. Microbiol. Immunol. 199:237-274). Upregulation of this region and subfragments thereof has been shown to prevent or decrease the immune response to virally infected cells, leading to longer term gene expression. (Ilan, *et al.* (1997) PNAS 94:2587:2592, Bunder, *et al.* (1997) J. Virol. 71:7623-28). Therefore, modifications to the E3 region (or sub-components thereof) to overexpress their proteins (e.g. by upregulating the E3 region using a strong constituitive promoter such as CMV) may be desirable to allow for a greater degree of viral replication due to its ability to avoid or delay the immune mediated clearance of infected cells.

B. Targeting Modifications:

The present invention provides recombinant viruses which contain "targeting modifications" in order to achieve preferential targeting of the virus to a particular cell type. The term "targeting modification" refers to modifications to the viral genome designed to result in preferential infectivity of a particular cell type. Cell type specificity or cell type targeting may also be achieved in vectors derived from viruses having characteristically broad infectivities such as adenovirus by the modification of the viral envelope proteins. For example, cell targeting has been achieved with adenovirus vectors by selective modification of the viral genome knob and fiber coding sequences to achieve expression of modified knob and fiber domains having specific interaction with unique cell surface receptors. Examples of such modifications are described in Wickham, *et al.* (1997) J. Virol 71(11):8221-8229 (incorporation of RGD peptides into

lines with varying genotype as well as increased expression levels relative to replication deficient viruses expressing p53 (ACN53).

In one experiment, the expression of p53 from the FAMA vector was compared to a substantially similar vector wherein p53 was operably linked to the CMV constituitive promoter. The experiment was conducted in substantial accordance with the teaching of Example 2.c. herein and the results are presented in Figure 4 of the attached drawings. his demonstrates that the FAMA virus does replicate in a temporal manner compared with the CMV containing FAIC virus.

In an additional expreiment conducted in accordance with Example 2.d., viral replication from FAMA was compared to the substantially similar FAIC vector where the temporal major late promoter was replaced by the constituitive CMV promoter. By expressing the p53 protein from the MLP promoter in a temporal manner, a greater replication of the virus is achieved relative to a constitutive promoter. The results are presented in Figure 5 of the attached drawings.

C. *In vivo* Experiments:

In order to confirm the efficacy of the vectors of the present invention *in vivo* in an animal, the vectors of the present invention were evaluated in a mouse human prostate cancer model. The model and experiments were peformed in substantial accordance with the teaching of Example 3 herein. The data is presented in Table 1 below and in Figure 6 of the attached drawings.

Table 1. Tumor Volume Following *In vivo* Administration of FAMA

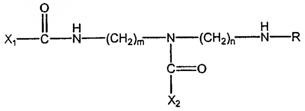
Virus	Avg. Tumor Volume (mm^3) ± SD[1]	% T/C[2]	# Animals Tumor Free
Saline control	1266 ± 403	100	0/6
Wt Ad 5	16	1.3	5/6
cZAZA	39 ± 13	3.1	3/6
cFAMA	25	2.0	5/6
cFAIC	16	1.3	5/6
FTCB	215 ± 93	17	0/6
ZZCB	702 ± 109	55	0/6

[1] Day 27 Post Initiation of Treatment
[2] Treated/saline control

B. Delivery Enhancing Agents:

The present invention further provides pharmaceutical formulations of the vectors of recombinant adenoviruses of the present invention with a carrier and a delivery enhancing agent(s). The terms delivery enhancers" or "delivery enhancing agents" are used interchangeably herein and includes one or more agents which facilitate uptake of the virus into the target cell. Examples of delivery enhancers are described in co-pending United States Patent Application Serial No. ________ filed July 7, 1998. Examples of such delivery enhancing agents include detergents, alcohols, glycols, surfactants, bile salts, heparin antagonists, cyclooxygenase inhibitors, hypertonic salt solutions, and acetates. Alcohols include for example the aliphatic alcohols such as ethanol, N-propanol, isopropanol, butyl alcohol, acetyl alcohol. Glycols include glycerine, propyleneglycol, polyethyleneglycol and other low molecular weight glycols such as glycerol and thioglycerol. Acetates such as acetic acid, gluconic acid, and sodium acetate are further examples of delivery-enhancing agents. Hypertonic salt solutions like 1M NaCl are also examples of delivery-enhancing agents. Examples of surfactants are sodium dodecyl sulfate (SDS) and lysolecithin, polysorbate 80, nonylphenoxypolyoxyethylene, lysophosphatidylcholine, polyethyleneglycol 400, polysorbate 80, polyoxyethylene ethers, polyglycol ether surfactants and DMSO. Bile salts such as taurocholate, sodium tauro-deoxycholate, deoxycholate, chenodesoxycholate, glycocholic acid, glycochenodeoxycholic acid and other astringents such as silver nitrate may be used. Heparin-antagonists like quaternary amines such as protamine sulfate may also be used. Cyclooxygenase inhibitors such as sodium salicylate, salicylic acid, and non-steroidal antiinflammatory drug (NSAIDS) like indomethacin, naproxen, diclofenac may be used.

Delivery-enhancing agents includes compounds of the Formula I:



wherein n is an integer from 2-8, X1 is a cholic acid group or deoxycholic acid group, and X2 and X3 are each independently selected from the group consisting of a cholic acid group, a deoxycholic acid group, and a saccharide group. At least one of X2 and

inhibitory activity; United States Patent No. 5,663,294 entitled Calpain-inhibiting peptide analogs of the kininogen heavy chain; United States Patent No. 5,661,150 entitled Drug for neuroprotection; United States Patent No. 5,658,906 entitled Cysteine protease and serine protease inhibitors; United States Patent No. 5,654,146 entitled Human ice homolog; United States Patent No. 5,639,783 entitled Ketone derivatives; United States Patent No. 5,635,178 entitled Inhibition of complement mediated inflammatory response using monoclonal antibodies specific for a component forming the C56-9 complex which inhibit the platelet or endothelial cell activating function of the C56-9 complex; United States Patent No. 5,629,165 Neural calcium-activated neutral proteinase inhibitors; United States Patent No. 5,622,981 entitled Use of metabotropic receptor agonists in progressive neurodegenerative diseases; United States Patent No. 5,622,967 entitled Quinolone carboxamide Calpain inhibitors; United States Patent No. 5,621,101 entitled Protein kinase inhibitors for treatment of neurological disorders; United States Patent No. 5,554,767 entitled Alpha-mercaptoacrylic acid derivatives having calpain inhibitory activity; United States Patent No. 5,550,108 entitled Inhibition of complement mediated inflammatory response; United States Patent No. 5,541,290 entitled Optically pure calpain inhibitor compounds; United States Patent No. 5,506,243 entitled Sulfonamide derivatives; United States Patent No. 5,498,728 entitled Derivatives of L-tryptophanal and their use as medicinals; United States Patent No. 5,498,616 entitled Cysteine protease and serine protease inhibitors; United States Patent No. 5,461,146 entitled Selected protein kinase inhibitors for the treatment of neurological disorders; United States Patent No. 5,444,042 entitled Method of treatment of neurodegeneration with calpain inhibitors; United States Patent No. 5,424,325 entitled aminoketone derivatives; United States Patent No. 5,422,359 entitled α-aminoketone derivatives; United States Patent No. 5,416,117 entitled Cyclopropenone derivatives; United States Patent No. 5,395,958 entitled Cyclopropene derivatives; United States Patent No. 5,340,922 entitled Neural calcium-activated neutral proteinase inhibitors; United States Patent No. 5,336,783 entitled Calpain inhibitor cystamidin A and its production; United States Patent No. 5,328,909 entitled Cyclopropenone derivatives; and United States Patent No. 5,135,916 entitled Inhibition of complement mediated inflammatory response. The uses of calpain inhibitors in gene therapy protocols is further described in Atencio, *et al.*, co-pending United States Patent Applications Serial Nos. 09/172,685 and 60/104,321 filed October

In the preferred practice of the invention as exemplified herein, a recombinant adenovirus containing a deletion of the E1B-55K gene function and expressing a tumor suppressor gene from the adenoviral major late promoter is formulated with a pharmaceutically acceptable carrier for administration by intravenous, intraperitoneal, or intratumor injection. The appropriate dose and method of administration of the vector to be administered to the mammalian organism in need of treatment will be determined by the skilled artisan taking into account the extent of metastatis of the primary tumor, the delivery enhancer(s) included in the formulation, the extent to which the immunological response is suppressed, etc. Each of these latter factors will decrease the dosage of the vector provided to the mammalian organism in need of treatment. In the preferred practice of the invention, a dosage of approximately 1×10^5 to 1×10^{13} particles (preferably 1×10^6 to 1×10^{11} particles, most preferably $x\ 10^7$ to 1×10^{10} particles) will be administered to the mammalian organism in one or more dosages in a treatment regimen. The typical course of treatment will be the daily administration of a pharmaceutically acceptable formulation of the vector of the present invention over a period of three to ten days, preferably five to eight days. In a preferred embodiment, the tumor suppressor gene is the wild-type p53 gene. In another preferred embodiment, the tumor suppressor gene encodes is a VP22-p53 fusion protein.

In a further preferred practice of the invention, the pharmaceutically acceptable carrier contains a delivery enhancing agent. In a further preferred practice of the invention, the delivery enhancing agent is a calpain inhibitor. In the most preferred practice of the invention as exemplified herein, the recombinant adenoviral vector E1B-dl55K-MLP-p53 is formulated in a carrier solution further comprising the calpain inhibitor n-acetyl-leu-leu-norcinal (calpain inhibitor 1) at a concentration of from approximately 1 to 50 micromolar. In such instances, the daily dosage may be reduced as compared to a formulation absent such delivery enhancing agents by a factor of one to two logs.

Preferably one employs an adenoviral vector endogenous to the mammalian type being treated. Although it is generally favored to employ a virus from the species to be treated, in some instances it may be advantageous to use vectors derived from different species which possess favorable pathogenic features. For example, it is reported (WO 97/06826 published April 10, 1997) that ovine adenoviral vectors may be used in

promoter. In the preferred practice of the invention, the immunosuppressive agent is etoposide and is administered daily for a period of from about 1 to 7 days (preferably 3-7 days) prior to administration of the vector. In such instances, the daily dosage in the course of treatment is reduced in comparison to those dosages provided absent such immunosuppressive agents.

The present invention also provides a method of ablating neoplastic cells in a population of normal cells contaminated by said neoplastic cells *ex vivo* by the administration of a recombinant adenovirus of the present invention to said population. An example of the application of such a method is currently employed in *ex vivo* applications such as the purging of autologous stem cell products commonly known as bone marrow purging. The term "stem cell product" refers to a population of hematopoietic, progenitor and stem cells capable of reconstituting the long term hematpoietic functionvof a patient who has received myoablative therapy. Stem cell products are conventionally obtained by apheresis of mobilized or non-mobilized peripheral blood. Apheresis is conventionally achieved through the use of known procedures using commercially available apheresis apparatus such as the COBE Spectra Apheresis System, commercially available from COBE International, 1185 Oak Street, Lakewood, CO. It is preferred that treatment conditions be optimized to achieve a "3-log purge" (i.e. removal of approximately 99.9% of the tumor cells from the stem cell produce) and most preferably a "5-log purge" (removal of approximatley 99.999% of tumor cells from the stem cell product). In the preferred practice of the invention, a stem cell product of 100 ml volume would be treated at a concentration of from about $1x10^6$ to $1x10^{10}$ particles/ml of the recombinant adenovirus of the present invention for a period of approximately 4 hours at 37°C.

B. Recruitment of Dendritic Cells:

The present invention provides a recombinant viral vectors capable of recruiting immature dendritic cells to a tumor site and exposing the dendritic cells to a localized high concentration of tumor antigens characteristic of the tumor present in the patient. The vectors of the present invention are specifically engineered to induce killing of tumor cells. The lysed tumor cell (or the apoptotic bodies produced by an apoptosed tumor cell) provides a rich localized concentration of tumor specific proteins. By introducing a gene encoding a dendritic cell chemoattractant, immature dendritic cells

systems (MRI). Alternatively, such vectors may also be employed to express a cell surface protein capable of recognition by a binding molecule such as a fluorescently labelled antibody. Examples of *in vivo* applications include imaging applications such as X-ray, CT scans or Magnetic Resonance Imaging (MRI).

X. Method of Making The Compositions:

The present invention further provides a method of producing the recombinant adenovirus comprising the modifications to packaging domains described above, said method comprising the steps of:

a. infecting a producer cell with a recombinant virus;

b. culturing said infected producer cell under conditions so as to permit replication of the viral genome in the producer cell;

c. harvesting the producer cells, and

d. purifying the recombinant adenovirus.

The term "infecting" means exposing the recombinant adenovirus to the producer cell under conditions so as to facilitate the infection of the producer cell with the recombinant virus. In cells which have been infected by multiple copies of a given virus, the activities necessary for viral replication and virion packaging are cooperative. Thus, it is preferred that conditions be adjusted such that there is a significant probability that the producer cells are multiply infected with the virus. An example of a condition which enhances the production of virus in the producer cell is an increased virus concentration in the infection phase. However, it is possible that the total number of viral infections per producer cell can be overdone, resulting in toxic effects to the cell. Consequently, one should strive to maintain the infections in the virus concentration in the range of 10^6 to 10^{10}, preferably about 10^9, virions per ml. Chemical agents may also be employed to increase the infectivity of the producer cell line. For example, the present invention provides a method to increase the infectivity of producer cell lines for viral infectivity by the inclusion of a calpain inhibitor. Examples of calpain inhibitors useful in the practice of the present invention include calpain inhibitor 1 (also known as N-acetyl-leucyl-leucyl-norleucinal, commercially available from Boehringer Mannheim).

the producer cell line, oxygen concentration is preferably maintained from approximately 50% to approximately 120% dissolved oxygen, preferably 100% dissolved oxygen. When the concentration of viral particles (as determined by conventional methods such as HPLC using a Resource Q column) begins to plateau, the reactor is harvested.

The term "harvesting" means the collection of the cells containing the recombinant adenovirus from the media. This may be achieved by conventional methods such as diffential centrifugation or chromatographic means. At this stage, the harvested cells may be stored or further processed by lysis and purification to isolate the recombinant virus. For storage, the harvested cells should be buffered at or about physiological pH and frozen at -70C.

The term "lysis" refers to the rupture of the producer cells. Lysis may be achieved by a variety of means well known in the art. When it is desired to isolate the viral particles from the producer cells, the cells are lysed, using a variety of means well known in the art. For example, mammalian cells may be lysed under low pressure (100-200 psi differential pressure) conditions or conventional freeze thaw methods. Exogenous free DNA/RNA is removed by degradation with DNAse/RNAse.

The term "purifying" means the isolation of a substanially pure population of recombinant virus particles from the lysed producer cells. Conventional purification techniques such as chromatographic or differential density gradient centrifugation methods may be employed. In the preferred practice of the invention, the virus is purified by column chromatography in substantial accordance with the process of Huyghe *et al.* (1995) *Human Gene Therapy* 6: 1403-1416 as described in co-pending United States Patent application Serial No. 08/400,793 filed March 7, 1995.

Additional methods and procedures to optimize production of the recombinant adenoviruses of the present invention are described in co-pending United States Patent Application Serial No. 09/073,076, filed May 4, 1998.

The purified virus is then admixed with appropriate excipients and carriers or delivery enhancing agents. The solution is sterilized for individual packaging and vialed for storage.

by restriction enzyme analysis to confirm the desired mutations.

This procedure was used to introduce restriction enzyme nuclease cleavage sites in the E1B 55K coding region. The first site was introduced by modifying positions 2247 and 2248 of the wild type Ad5 genome wherein a guanine^{2247} was replaced with a thymidine and thymidine^{2248} replaced with cytosine (respectively) to introduce a EcoR1 cleavage site. This results in a modification of the E1B coding sequence at position 77 from valine to serine. A second resriction site was introduced at position 3272 wherein thymidine^{3272} was replaced with cytosine site (silent mutation) to introduce an XhoI site. The new restriction enzyme sites were used in a restriction enzyme digest with EcoRI and Xho1.

A cassette containing the p53 coding sequence, under control of the adenovirus Major Late Promoter and tripartite leader sequence, was removed by EcoR1 and partial Xho1 digestion from the plasmid, pAd-MLP-p53. This plasmid is based on the pBR322 derivative pML2 (pBR322 deleted for base pairs 1140-2490) and contains an adenovirus type 5 sequences extending from base pair 1 to 5788 except that it is deleted for adenovirus type 5 base paris 357-3327. At ths site the Ad5 357-3327 deletion, a transciption unit is inserted iwhich is comprised of the adenovirus type 2 major late promoter, the adenovirus type 2 tripartite leader DNA and human p53 cDNA. This EcoRI/Xho1 fragment is inserted into the EcoR1 and Xho1 sites introduced into the E1B55k coding region. The polyA sequence that follows wtAd5 pIX was amplified by PCR (Ad5 sequence 4001-4368). The primers used for amplification included sequences to introduce an EcoR1 site at the 5' end of the Ad5 sequence, and a SacII site at the 3' end of the Ad5 sequence. This fragment was then inserted into the EcoR1-SacII sites immediately following the E1B19k coding sequence. The SacII site was included in the MLP-p53 cassette inserted previously, and was upstream of the MLP promoter sequence. The resulting E1B mutation results in a sequence encoding the first 76 amino acids of the E1B55K protein followed by 11 missense amino acids resulting in a non-functional deleted E1B protein.

Construction of the E1B<u>dl</u>55K-MLP-p53 adenovirus was carried out by using homologous recombination in the adenovirus E1-region containing 293 cell line by the method of McGory, *et al.*, (1988) Virology 163, 614-617. This method requires two fragments of DNA, one a transfer plasmid containing the E1B55k deleted/MLP-p53 cassette and the other Ad5 viral DNA containing the wtAd5 genome ("Ad5 large

Example 2.b. p53 Expression SK-HEP1 Cells

This evaluation was performed in substantial accordance with the teachining of Example 2.a above except that the experiment was conducted in SK-HEP1 hepatocellular carcinoma cells and the six well plate was seeded with 7.5×10^5 cells per well. The results are presented in Figure 2 of the attached drawings.

Example 2.c. p53 Expression NCI H358 Cells

This evaluation was performed in substantial accordance with the teachining of Example 2.a above except that the experiment was conducted in NCI H358 cells except that the six well plate was seeded with 1.5×10^5 cells per well. The cells were infected as in Example 2.a. above using 1.8×10^9 particles/ml concentration of the indicated viruses for Figure 3 and 1.8×10^8 for Figure 4. The results are presented in Figure 3 and 4 of the attached drawings.

Example 2.d. Time Course of Viral Replication

SK-BR3 cells were infected with a one hour pulse of the following recombinant adenoviral ectors at a concentration of 1.8×10^9 particles/ml:

1. Mock: non-infected cells
2. rAdcon: a recombinant adenovirus lacking E1 and protein IX function without a p53 coding sequence (Wills, et al.)
3. E1Bdl55K: A recombinant adenovirus containing the E1B-55K deletion described in Example 1 above with no exogenous transgene cassette.
4. 55K/MLPp53: The recombinant adenovirus cFAMA prepared in substantial accordance with the teaching of Example 1 above.
5. rAd-p53: ACN53 (Wills, et al.)
6. 55K/CMVp53: A recombinant adenovirus cFAIC containing the E1B-55K deletion described above further comprising an expression cassette encoding p53 under control of the CMV promoter
7. Ad5WT: Wild type adenovirus type 5.

The cells were harvested at approximately 48 hours post infection. The DNA was applied to a agarose gel and stained with ethidium bromide according to techniques well known in the art. The results are presented in Figure 5 of the attached drawings.

Example 3. Demonstration of Therapeutic Efficacy *In vivo*

PC-3 cells (prostate carcinoma, p53 null) were injected subQ into flanks of nude mice. When tumors were palpable (day 11) , virus was intratumorally injected for 5 consecutive days at a dose of 1×10^{10} particles per injection on days 11-15 post PC-3

oligonucleotide site directed technique of Zoeller and Smith (1984) *DNA* 3, 479-488, as modifed by Kunkel (1985) *PNAS* 82, 488-492. All of the reagents, bacterial strains, and M13 vectors used for mutagenesis are provided in the Muta-Gene *in vitro* mutagenesis kit (commercially availble from Bio Rad, Hercules, CA). The M13 template DNA, useful for mutagenesis of the E1A region, contains Ad5 sequences from nucleotide positions 22-1339 inserted between the BamH1 and Xba1 restriction enzyme sites in the multiple cloning sequence of M13mp19. The resulting bacteriophage construct, M13mp19E1A, is then propagated in *dut ung E. coli* bacterial strain CJ236 which results in an occasional incorporation of uracil in place of thymidine in the newly synthesized DNA. The oligonucleotides, for construction of the E1 mutants, are synthesized to consist of sequences of either 11 or 12 nucleotides of Ad5 sense DNA on either side of the sequence that was to be removed.

For the mutagenesis reaction, the mutagencic oligonucleotides are first phosphorylated at the 5' end, and then annealed to uracil containing M13mp11E1A single-stranded template DNA. The annealed primer/template reactions are incubated with T4 DNA polymerase, T4 DNA ligase and deoxyribonucleotides (dATP, dCTP, dGTP and dTTP) to synthesize a complementary strand containing the E1A mutation of interest. The complementary strand synthesis reaction are then transfomed into the *ung*$^+$ wild type host bacterial strain, MV1190. After transformation the parental M13mp19E1A DNA strand, which contains uracil, cannot be replicated efficiently in MV1190. Therefore the replicative form double strand DNA containing the E1A mutation of interest is enriched. M13mp19E1A phage DNA from potential E1A mutants is first screened by restriction enzyme analysis and then by DNA-sequencing, in both strands, to confirm the desired E1A-mutations.

Construction of an adenovirus comprising the E1Adl01/deletions is carried out by using homologous recombination in the adenovirus E1-region containing 293 cell line by the method of McGory, *et al.*, (1988) Virology 163, 614-617. This method requires two plasmids, one a viral plasmid containing the entire wtAd5 genome modified as in Example 1 to contain the E1bdl55K deletion and the MLP promoter (pXC1-E1B55-2A), and the other a transfer plasmid containing an E1A gene with the dl01/07 double E1A-mutant . The transfer plasmid, pLE2 contains wtAd5 sequences from 22-1774 cloned in the tetracycline gene of pBR322 Jelsma *et al.*, (1988)*Virology* 163, 494-502. For transfer of the E1A dl1101 and dl1107 E1a-mutants from the

Claims

I claim:

1. A replication competent recombinant virus containing a therapeutic transgene operably linked to a late regulatory element.
2. The virus of claim 1 wherein the virus is an adenovirus.
3. The virus of claim 2 wherein the late regulatory element is the adenoviral major late promoter.
4. The virus of claim 3 wherein the therapeutic transgene is a tumor suppressor gene.
5. The virus of claim 4 wherein the tumor suppressor gene is p53.
6. The virus of claim 5 further comprising a deletion of E1B-55K function.
7. The virus of claim 6 further comprising a replication control sequence operably linked to an early gene.
8. The virus of claim 7 wherein the replication control sequence is a tumor specific promoter.
9. The virus of claim 7 wherein the replication control sequence is the alpha-fetoprotein.
10. The virus of claim 9 wherein the early gene is the E4 gene.
11. The virus of claim 6 further comprising a deletion of E1a 12S and 13S functions. (01/07)
12. A pharmaceutical formulation comprising a replication competent recombinant virus containing a therapeutic transgene operably linked to a late regulatory element and a pharmaceutically acceptable carrier.
13. The formulation of Claim 12, further comprising a delivery enhancing agent.
14. The formulation of claim 13 wherein the delivery enhancing agent is a calpain inhibitor.
15. The formulation of claim 14 wherein the calpain inhibitor is N-acetyl-leu-leu-norcinal.
16. The formulation of Claim 13 wherein said delivery enhancing agent is a detergent.

INTERNATIONAL SEARCH REPORT

Inte...ational Application No
PCT/US 99/26004

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/86 C12N15/57 C07K14/47 A61K48/00 A61P35/00
C12N5/06 C12N5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WILLS K N ET AL: "DEVELOPMENT AND CHARACTERIZATION OF RECOMBINANT ADENOVIRUSES ENCODING HUMAN P53 FOR GENE THERAPY OF CANCER" HUMAN GENE THERAPY,XX,XX, vol. 5, no. 9, 1 September 1994 (1994-09-01), pages 1079-1088, XP000579605 ISSN: 1043-0342 cited in the application	1-6
Y	abstract; figure 1 page 1081, right-hand column, paragraph 2	11
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 April 2000	08/05/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Mateo Rosell, A.M.

1

International application No.

INTERNATIONAL SEARCH REPORT

PCT/US 99/26004

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 18,19
are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.